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Edited by

C. E. Cornelius
Department of Physiological Sciences
School of Veterinary Medicine
University of California
Davis, California

Michael Burridge
Department of Infectious Diseases
College of Veterinary Medicine
University of Florida
Gainesville, Florida

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Edited by

James L. Bittle

Department of Molecular Biology
Scripps Clinic and Research Foundation
La Jolla, California

and

Frederick A. Murphy

Center for Infectious Diseases
Centers for Disease Control
Atlanta, Georgia

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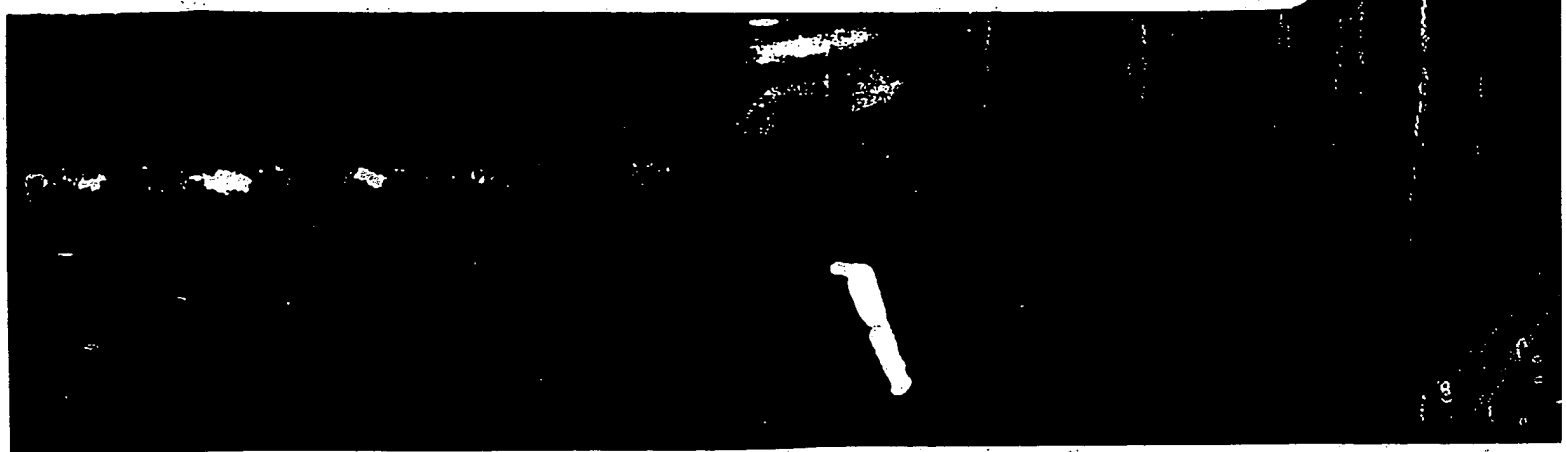

Infectious Recombinant Vectored Virus Vaccines

JOSEPH J. ESPOSITO AND FREDERICK A. MURPHY

Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia

I. Introduction

A. Historical Perspective

Live-virus immunization was practiced as early as the tenth century in China and India; pustular fluid from a patient with smallpox was inoculated into previously uninfected persons. The practice, which we now call variolation, caused disease of varying severity and 1–2% mortality, in contrast to the 25% mortality of natural smallpox infection. By the mid-1700s, the practice had become widespread in many parts of the world. Later in that century, the English country physician, Edward Jenner, observed that variolation did not "take" in persons who previously had been infected with the pox from the teats of cows. In 1796, Jenner inoculated an 8-year-old boy with pustular fluid from a milkmaid's pox lesion. Within a few days an erythematous lesion appeared at the inoculation site and then regressed. Six weeks later Jenner challenged the boy with smallpox pustular fluid; a lesion developed but no disease followed. Jenner successfully repeated the experiment and further showed that the protective element in pus could be maintained by arm-to-arm passage. For almost 200 years the practice (originally called "cowpoxing") spread throughout the world, eventually confirming Jenner's prediction that smallpox could be eliminated. In 1840, Adelchi Negri, in Italy, began maintaining the cowpoxing inoculum in calves. In 1881, Louis Pasteur proposed the term "vaccination" instead of "inoculation," honoring Jenner's discovery; hence, the inoculum became known as vaccinia virus.

From the experiments of Jenner through the development of live-virus vaccines against yellow fever (Theiler and Smith, 1937), poliomyelitis (Sabin, 1955), measles (Enders *et al.*, 1960), and other diseases, vaccine viruses have been derived empirically, usually by repeated passages in animals, embryonating eggs, or cell cultures. The results represent great success stories—the live-virus vaccines have had excellent records of efficacy, reasonable safety, and modest cost. Generally, live-virus vaccines have provided long-lasting protection. Even when immunity levels have fallen with time, natural subclinical reinfections have served as harmless boosters. Inherently, both cell-mediated and humoral immunity have usually been stimulated by live-virus vaccines, just as in natural infections. Despite these and other qualities, live-virus vaccines do have disadvantages, and in many cases some researchers believe that a point of diminishing returns has been reached in regard to new developments. Some also believe that

other approaches must be tried if we are to make significant further improvements in viral vaccines.

In the past two decades, technologies for biochemical characterization and genetic manipulation of viruses have led to the development of novel mutant and recombinant viruses. For example, novel deletion mutants of pseudorabies virus, derived by genetic engineering, have proven to be nonpathogenic yet effective in protecting swine from wild-type virus challenge (Kit *et al.*, 1987; Quint *et al.*, 1987). The overall promise of deletion mutants seems limited, however, especially relative to the promise of insertion mutants. In some ways one particular gene insertion strategy, that of heterologous gene insertion into infectious viruses which are then used as vectors, seems most promising of all.

B. The General Context for Vectored Virus Vaccines

Two classes of viral vectors may be differentiated. One class comprises replication-defective vector viruses that produce infectious progeny only via complementation by specifically transformed cells (SV40, adenoviruses) or by helper-virus superinfection (retroviruses). The second class comprises replication-competent viruses. Herpesviruses, adenoviruses, and poxviruses are the most notable examples of viruses that can serve as vectors for heterologous genes while maintaining full infectivity. Studies involving both classes of altered viruses have increased our insight into genomic structure, gene regulation, and transcription and translation, and have furthered our understanding of disease mechanisms and host defenses.

Experimental immunizations with infectious virus vectors carrying one or more heterologous genes for immunogenic proteins have been remarkably successful in experimental animals systems. Such immunizations have enabled the presentation of immunogenic proteins to the host immune system in an authentic way, mimicking closely the way antigens are presented in natural infections. Such successes have vaulted infectious virus vectors into consideration as vaccines for humans and animals.

Clearly, however, the use of poxviruses, herpesviruses, and adenoviruses as candidate vectors is not without problems. They all have complex *in vivo* replication demands, less-than-perfect virulence characteristics, and some undesirable host range traits. They all require further research to define and optimize these characteristics. For example, the persistence of adenoviruses in lymphatic tissues and herpesviruses in neural tissues and the progressive course of vaccinia virus

infection in immunocompromised individuals must be studied further. We must better understand the consequences of specific deletion mutations in these vector viruses, such as those caused during the insertion of heterologous DNA. Such mutations may affect virulence, tropism, and host range, as well as immunogenicity.

In considering development and use of infectious vectored virus vaccines, we must be aware of the differences between human and veterinary vaccine use. In the case of human vaccines, the central focus is upon safety. Not only are adverse vaccine reactions objectionable, but they can be the basis for legal liability claims that influence all other aspects of vaccine development and use. In the case of veterinary vaccines, the central focus is upon cost-effectiveness in the context of an overall production–economy equation. There is much less regulatory control over the manufacturing and the quality assurance of animal vaccines as compared to human vaccines. The regulatory infrastructure for human vaccines enjoys a credible national and international reputation with leadership provided through the activities of the U.S. Food and Drug Administration (FDA) and the World Health Organization (WHO).

This chapter highlights aspects of infectious vectored viruses for human and animal vaccines, using the vaccinia virus system as the principal example. Several reviews of this subject have recently been published (Moss *et al.*, 1983; Smith and Moss, 1984; Smith *et al.*, 1984a,b; Quinnan, 1985; Mackett *et al.*, 1985a; Paoletti *et al.*, 1985a,b; Flexner, 1985a; Mackett and Smith, 1986; Moss and Flexner, 1987; Piccini and Paoletti, 1986, 1988; Beaud *et al.*, 1987). The initial success with vaccinia virus as a gene expression vector has now also begun to be extended to other poxviruses, notably fowlpox. Because licensing and use of such vaccines will require rigorous evaluation and efficacy and safety requiring new testing procedures and new regulations, we have approached the subject accordingly. We describe the biological, molecular, and immunological characteristics of vaccinia virus and other viruses in regard to their use as vectored viral vaccines, and we summarize the technology of heterologous gene transfer into vaccinia virus. We describe the nature of the infection caused in humans and animals by standard vaccinia virus, as the basis for study of infections caused in experimental animals by vectored vaccinia virus vaccine candidates. We note areas in need of further research and further policy development. All in all, we try to point out the great promise of this vaccine technology in advancing our capability for controlling diseases of humans and animals in the future.

II. Characteristics of Poxviruses as Vectors

A. Poxviruses with Potential for Use as Vectors

Over many years much experience has been gained with vaccines against human and animal poxvirus diseases. A massive amount of information on vaccinia virus (smallpox) vaccine in humans was gathered during the WHO Smallpox Eradication Programme; this included information on clinical, laboratory, and epidemiological aspects of vaccine usage. In veterinary medicine, poxvirus vaccines have been used to protect many species of animals and birds. These include vaccinia or ectromelia viruses to prevent mousepox and vaccinia virus to prevent rabbitpox and other orthopoxvirus infections of captive and domestic animals. Attenuated fowlpox virus vaccines have been used to protect poultry. Attenuated autologous viruses have been used for vaccinating against goatpox, sheeppox, and lumpy skin diseases. Attenuated myxoma and fibroma virus vaccines have been developed for domestic rabbits. Also, over many years, vaccines have been made from lesions of naturally infected animals (for use similar to variolation in humans) to prevent camelpox and contagious ecthyma (orf) of sheep. Mild to severe infections of animal handlers have been related to the use of such contagious ecthyma "vaccines" (Fenner *et al.*, 1987; Tripathy *et al.*, 1981).

Any poxvirus can be considered as a potential vector for heterologous genes, depending upon particular valuable characteristics, such as a wanted host range. For example, a raccoon poxvirus, recovered from the lungs of healthy raccoons (Alexander *et al.*, 1972), has been used recently as a vector for the rabies virus glycoprotein gene (Esposito *et al.*, 1987, 1988). This vector was developed for use as an oral, bait-delivered vaccine to combat the raccoon rabies outbreak that spread throughout the mid-Atlantic area of the United States during the 1980s (Jenkins and Winkler, 1987; Centers for Disease Control, 1987). Captive raccoons, fed the infectious recombinant virus in bait, have produced high levels of rabies-neutralizing antibodies and were protected against a lethal virus challenge. Safety and further efficacy studies are being planned to pursue field trials.

B. Lessons from the Smallpox Eradication Programme

The major factor in the success of the WHO Smallpox Eradication Programme was the development of a unique international infrastruc-

ture, which served as the focus for cooperative clinical, laboratory, and epidemiological research, as well as for vaccine delivery worldwide. Nevertheless, the WHO Programme could not have been successful if it were not for particular characteristics of the etiologic agent, variola virus, and of the disease, smallpox:

1. Smallpox was greatly feared, fostering universal interest and support for the use of a vaccine.
2. Smallpox was easily diagnosed clinically, allowing a focused use of the vaccine.
3. Variola virus did not persist in infected individuals nor in the environment, allowing a one-time sweep by vaccinators without concern for covering individuals missed or born later.
4. Variola virus was transmitted only by relatively close contact, allowing vaccinators to keep up with virus movement and allowing quarantine to be used in certain settings.
5. Variola virus was not transmitted by arthropods, allowing the use of "ring-vaccination" selective containment protocols in the late stages of the WHO Programme.
6. Variola virus had no animal reservoir and no capacity to persist in a zoonotic cycle.

C. Advantages of Vaccinia Virus as a Vaccine and as a Vector

In addition to those characteristics of variola virus and the disease smallpox that favored eradication, several attributes of vaccinia virus vaccine itself contributed to the success of the WHO Programme, particularly in developing countries:

1. Vaccinia virus vaccine is easily administered in a single-dose regimen.
2. Vaccinia virus vaccine is simple to manufacture and inexpensive to test. This is crucial—much of the cost of a vaccine is related to the complexity of production and related costs of quality assurance and safety testing.
3. Vaccinia virus vaccine is very stable. This is an outstanding quality of the virus itself (freeze-dried vaccine withstands 37°C for 30 days).
4. Vaccinia virus vaccine induces a relatively long-lived immunity.

These properties (see also Fenner, 1985a; Henderson and Arita, 1985) of vaccinia virus vaccine also can be taken as a list of advantages for using vaccinia virus and other poxviruses as vectors for human and animal vaccines. Of course certain poxviruses, such as avian poxviruses and myxoma virus, which are arthropod-borne in nature, would be subject to particular additional constraints.

Vaccinia virus vaccine had been very acceptable to people throughout the world because of its great reputation for having brought about the eradication of one of the great plagues of mankind, smallpox. Certainly, the equation of benefit : cost : risk was balanced in favor of its use to achieve smallpox eradication. It might be envisioned that there would be no preconceived bias against its use for another purpose in humans or animals. In many places, however, it has been years since vaccinia virus has been used, thus population re-education might be a significant factor. In some cases where conventional human or animal vaccines have proven inefficacious or too expensive, vaccinia virus-vectored vaccines might be assessed most favorably. The fact that a single vector can be designed to carry several heterologous genes for immunizing against several diseases might further influence public acceptance.

D. Disadvantages of Vaccinia Virus as a Vaccine and as a Vector

Vaccinia vaccination is not without its disadvantages in humans (see Table I) (Arita and Fenner, 1985).

E. Safety of Vaccinia Vaccines

Historically, trial and error guided the selection of the safest strains of vaccinia virus for vaccine production. In recent years, only a few strains have been used: Lister Institute (Lister), New York Board of Health (NYBH), Tian Tan, and Padwadangar. Because of the consideration of vaccinia virus as a vector, Arita and Fenner (1985) re-examined earlier data on the frequency of complications following smallpox vaccination. They collated rates of CNS complications and other adverse effects in seven European countries and the United States during different periods. They found that vaccines originating from either the NYBH or Lister strains produced the lowest rates of complications. Both of these strains were used extensively in developing countries, but little information is available about adverse effects in such settings. In the United States, England, and Wales (where these viruses were used widely) adverse effects occurred at low rates (Table II).

Criticism has occurred concerning the rigor used in confirm...

TABLE I

ADVERSE REACTIONS FOLLOWING VACCINIA VACCINATION IN HUMANS

Reaction	Cause	Occurrence	Mortality rate
Exanthematous disease	Progressive vaccinia resulting from compromised cell-mediated immunity	Mostly in children; very rare	High
	Eczema vaccinatum occurring in individuals vaccinated while having eczema	Very rare	High
	Generalized vaccinia following hematogenous spread of virus with postules on many parts of body	Rare	No mortality
	Accidental infection of conjunctiva or other parts of body after scratching primary vaccination lesion	Transmission can occur by same means with same results	Very low
Central Nervous System (CNS) disease	Vaccinia encephalitis occurring unpredictably resulting in cerebral impairment, hemiplegia, convulsions, coma	Very rare	High
Other complications	Fetal vaccinia infection	Very rare	Varying severity; some mortality

etiology of these adverse effects. Progressive vaccinia infection and eczema vaccinatum are easily diagnosed clinically, but background intercurrent CNS disease is not clinically distinguishable, and specific etiologic confirming tests were not used. Therefore, it is likely that these data overestimate the risk of CNS disease caused by vaccinia virus.

TABLE II

FREQUENCY OF SERIOUS COMPLICATIONS OF VACCINATION IN THE UNITED STATES, ENGLAND, AND WALES, USING NEW YORK BOARD OF HEALTH AND LISTER VACCINES

Complication	Cases/million primary vaccinations[a]	
	Age < 1 year	Age ≥ 1 year
CNS disease	7–14	2.4–11.0
Progressive vaccinia	0–3	0.2–2.0
Eczema vaccinatum	4–8	6.0–10.0
Death	5–13	0.6–1.7

[a] Complications were much less frequent after revaccination. [From Fenner (1985a), reproduced with permission.]

Vaccinia virus, nonetheless, is associated with CNS complications. When Lister strain vaccine replaced other vaccine strains such as Hamburg, Berne, and Copenhagen in three European countries in the 1960s, the incidence of postvaccinal CNS disease dropped markedly (Arita and Fenner, 1985).

If vectored vaccinia virus vaccines are to be introduced for human use, a substantial reduction of adverse effects could be achieved with appropriate prevaccination screening. The Advisory Committee on Immunization Practices of the U.S. Public Health Service has recommended against vaccinating concurrently with other live-virus vaccines and against vaccinating high-risk individuals, such as persons with acute infections, eczema or other chronic dermatitides, pregnant women, children younger than 1 year of age, and immunocompromised persons. The latter includes persons with congenital or acquired defects in cell-mediated immunity such as severe combined immunodeficiency (dysgammaglobulinemia, Hodgkin's diseae, or acquired immunodeficiency syndrome (AIDS)), and persons receiving immunosuppressive drugs (alkylating agents, radiation, steroids, etc.). Even though today there is no reason for smallpox vaccination of general populations, these recommendations would serve well if infectious vectored vaccinia virus vaccines were to be introduced.

Veterinary use of vectored vaccinia virus vaccines would, of course, involve a minimum risk of human adverse effects. The principal risk would be from accidental inoculation, which would be of little consequence if vaccinators were prescreened and vaccinated accordingly. Also, deep intramuscular injection of animals instead of intradermal scarification might be useful in limiting virus transmission. Each vec-

tored recombinant to be considered as a vaccine candidate would have to be evaluated separately in regard to the risk it would pose to vaccinators and other animal handlers, however, no novel untoward effects would be expected.

F. Origins of NYBH and Lister Strains of Vaccinia Virus

Because the NYBH and Lister vaccinia virus strains have caused the lowest rates of adverse effects, they and possibly certain derivatives should be considered primary candidates for human and animal vectored virus vaccines. For this reason, a brief history of these viruses is presented.

The origin of vaccinia virus itself is obscure; it is not thought to be a naturally occurring virus, but rather a recombinant between variola virus and Jenner's cowpoxing virus or a descendant of a now-extinct poxvirus of horses that caused "grease" in Jenner's time. The lineage of Jenner's virus became further obscured as the virus was distributed worldwide via arm-to-arm passage and via passage in calves, sheep, and other animals. Often the inoculum would lose potency when it was mixed with variola pustular material. This practice continued into the early 1900s. Despite this history, vaccinia virus strains are remarkably similar; their DNA restriction profiles are quite similar and distinct from other orthopoxviruses (Esposito *et al.*, 1978, 1985; Esposito and Knight, 1985; Mackett and Archard, 1979).

The NYBH strain of vaccinia virus was established in 1856 from a British vaccine that originated before calf passage had replaced human arm-to-arm passage. It is regarded as one of the most direct descendants of Jenner's vaccine. At the New York City Board of Health laboratories, the virus was maintained by passage in the skin of rabbits and calves and occasionally by intradermal passage in humans. Passage in rabbit testes was practiced to decrease microbial contamination and revitalize potency. The NYBH strain was used for vaccine production by many manufacturers, including Wyeth Laboratories, Inc. This company has passaged the virus in calves since 1929, in later years producing the lyophilized product "Dryvax calf-lymph." Others using the NYBH strain as starting material were Connaught Laboratories, Inc., Lederle Laboratories, Inc., the Massachusetts Department of Health, and national laboratories in many countries.

The Lister strain is said to have been isolated in 1870 during the Franco–Prussian war from a Prussian soldier with smallpox. The strain, first produced at the Lister Institute at Elstree, has been used in Great Britain since 1892. This strain has probably had the widest distribution of any in the world under many synonyms, including

Liverpool, Elstree, Merieux-37, and Nigeria. The Padwadangar strain, used widely in India, is known to have been derived from a mixture of Lister strain and a strain of unknown origin (Kay, 1973; Marennikova *et al.*, 1969). Further information on vaccinia virus strains can be found in papers by Wokatsch (1972), Fenner (1958), Briody (1959), Ghendon and Chernos (1964), Quinnan (1985), and the proceedings of two symposia (Gusic, 1969; Regamy and Cohen, 1973).

In keeping with the modern "seed-virus" concept, standards for good manufacturing practices (GMPs), and U.S. Food and Drug Administration quality assurance provisions for live-virus vaccines (Code of Federal Regulations, Title 21), two laboratories involved in vectored vaccinia virus development have rederived Lister and NYBH vaccine strains from manufacturers' seed viruses (B. Moss and J. Dalrymple, personal communications).

III. General Characteristics of Poxviruses

In the following sections we consider poxvirus virion structure, virion morphogenesis, genome structure, gene expression, biological (phenotypic) markers, and immunobiology, since these characteristics relate to the use of poxviruses as vectors. Reviews of these subjects have been published (White and Fenner, 1986; Fenner *et al.*, 1987, 1988; Fenner and Nakano, 1988; Nakano and Esposito, 1988; Dumbell and Huq, 1986; Moss, 1985; Tripathy *et al.*, 1981; Moss *et al.*, 1983; Holowczak, 1982, 1983; Cole and Blanden, 1982; Wittek, 1982; McFadden and Dales, 1982; Fenner, 1985b; Dales and Pogo, 1981; Cho and Wenner, 1973; Briody, 1959).

A. Poxvirus Virion Structure

Poxvirus virions are structurally complex, brick-shaped, and approximately 300 × 240 × 100 nm in size (except for parapoxviruses, which are ovoid, 260 × 160 nm). Virions have four major components: a dumbell-shaped *core* that contains the DNA genome, *lateral bodies* lying in the concavities of the core, an *outer membrane*, and a surrounding *envelope* (Fenner *et al.*, 1987, 1988; Dales and Pogo, 1981). Two types of infectious virus particles have been recognized during growth of poxviruses: extracellular enveloped virions (EEVs) and intracytoplasmic naked virions (INVs).

EEVs, studied especially in vaccinia and cowpox virus infections, are composed of all four components. They are spontaneously released from infected cells and, depending on virus strain and cell type, may consti-

e up to 10% of the virus produced (Boulter and Appleyard, 1973). Vs are antigenically distinctive because of the composition of the velope, which is derived from modified plasma membrane. Vaccinia us EEV envelopes contain a 37-kilodalton (kD) palmitate-binding tein that constitutes 7% of the EEV protein mass and nine viral coproteins including an 85-kD protein component of the hemaggluti- (Hirt *et al.*, 1986; Payne, 1979, 1986; Shida, 1986a). The poxvirus naggiutinin is separate from virions; it consists of virus-modified l membranes. EEVs are involved in virus dissemination *in vivo* yne, 1980; Payne and Kristensson, 1985), and therefore are impor- t considering the use of vaccinia virus for infectious vectored virus cines. To illustrate, it has been suggested that the distinctive tigenicity of the envelope of EEVs directs tissue tropism, patho- nicity, immunogenicity of the envelope of EEVs directs tissue pism, pathogenicity, immunogenicity, and possibly host range perties. Therefore, insertion of heterologous proteins into the velope of EEVs present in vectored vaccinia virus vaccines might er vaccine properties in deleterious ways. This has not yet been own with vaccine candidates, but the nature and effect of heterolo- us proteins present in the envelope of recombinant poxvirus virions ast be examined.

INVs, the bulk of infectious particles produced in infected cells, are mposed of a core, lateral bodies, and an outer membrane. INVs are leased *in vivo* by cytolysis and in the laboratory by mechanical cell sruption. The location in the virion and the functional role (e.g., in sorption, penetration, fusion, etc.) of many vaccinia virus INV outer embrane and core proteins have been determined (Oie and Ichihashi, 37; Ichihashi and Oie, 1980; Dales and Pogo, 1981; Moss, 1985; nner *et al.*, 1987). The genes for some of these proteins have also been apped and sequences determined (Earl and Moss, 1987; McGeoch *et* , 1987; Rodriguez *et al.*, 1987). INVs of some poxviruses (cowpox, cromelia, raccoon poxvirus, fowlpox) can become occluded in and otected by intracytoplasmic type A inclusion bodies. Since inclusion dies might affect the stability and transmissibility of vectored pox- ruses, their presence and effect should be studied further. Nonspecific trapment of foreign gene products during maturation of vaccinia us also must be studied further (Franke and Hruby, 1987).

B. Poxvirus Replication

Poxvirus virion adsorption and penetration occur directly via plasma embrane fusion or indirectly via endophagocytosis. Virion outer embrane fusion and uncoating then occur concomitantly and the

virion core is released into the cytoplasm. Virion cores carry a complete transcription system that, during early stages of infection, produce mRNAs for at least 100 proteins, including the viral DNA polymerase and transcription enzymes. Soon after infection, a shutdown of cellular protein synthesis occurs, mediated in part by viral protein(s) and/or by production of 100- to 300-base polyadenylated nontranslated viral RNAs that affect ribosome function (Bablanian and Banerjee, 1986). Although vaccinia virus can replicate in enucleated cells (Villarreal *et al.*, 1984), its distinctive multisubunit RNA polymerase appears to contain host cell RNA polymerase II large-fragment activity (Morrison and Moyer, 1986; Broyles and Moss, 1986; Wilton and Dales, 1986).

Poxvirus replication can be divided into distinctive early and late stages that flank the peak of viral DNA replication. As viral DNA accumulates, transcriptional switch-over occurs and, in late stages, about 100 proteins, primarily virion structural proteins, are synthesized. Altogether, vaccinia virus encodes about 280 proteins, of which about 100 are structural (Carrasco and Bravo, 1986; Essani and Dales, 1979).

Poxvirus replication occurs in intracytoplasmic sites, termed "virus factories" (type B inclusion bodies). Virion morphogenesis becomes visible after the peak of DNA synthesis. Lipoprotein crescents (precursors of virion outer membrane structures) are formed, and these surround condensing viral genomes to form immature virions. Upon maturation, some virions migrate to the cell surface, undergoing a complex process of envelopment (Payne and Kristensson, 1985; Tsutsui *et al.*, 1983) and release as EEVs, but most remain as INVs until cytolysis occurs.

C. The Poxvirus Genome

The genomic DNA of poxviruses is a very large, linear molecule; it ranges in size from 110 to 150 kbp (parapoxviruses, capripoxviruses) to 165 to 210 kbp (orthopoxviruses) to 280 kbp (avipoxviruses). On the basis of DNA endonuclease studies, researchers know that cleavage sites within poxvirus genera are highly conserved among orthopoxviruses (Mackett and Archard, 1979; Esposito and Knight, 1985) and capripoxviruses (Black *et al.*, 1986), less so among leporipoxviruses (Block *et al.*, 1985), molluscipoxviruses (Porter and Archard, 1987), and yatapoxviruses (Esposito, unpublished data), and least so among parapoxviruses (Mercer *et al.*, 1987; Robinson *et al.*, 1987; Gassmann *et al.*, 1985) although there is considerable cross-hybridization within genera.

Both ends of poxvirus DNA are covalently closed, forming hairpin loops. In vaccinia and myxoma viruses, the loops are incompletely base-paired and exist in two equimolar isomeric forms that are inverted and complementary to each other (Upton *et al.*, 1987; Baroudy *et al.*, 1983; Pickup *et al.*, 1983). Extending from 1 to 40 kbp from the hairpin ends, depending on the virus strain, the terminal regions comprise inverted terminal repetitions (ITRs). Within vaccinia virus ITRs, 200 bp from each end, are tandem repetitions arrayed in adjacent sets (about 1 kbp each) that are separated by short intervening sequences (about 400 bp). DNA from highly passaged vaccinia virus strains and vaccinia vaccine stocks exhibits extensive terminal-length heterogeneity; this is caused by multiple recombinations that generate varying numbers and sizes of sets of tandem repeats (Wittek, 1982; DeLange and McFadden, 1987; Pickup *et al.*, 1982).

Significant mutational events, such as deletions, insertions, duplicative rearrangements of terminal regions (ITR copy transpositions), and various recombinatorial effects (e.g., nonhomologous crossover, random nonreciprocal transfer of controlling and/or coding sequences), occur spontaneously during poxvirus DNA replication (Pickup *et al.*, 1984; Moyer *et al.*, 1980; Esposito *et al.*, 1981; Archard *et al.*, 1984; Macaulay *et al.*, 1987). Such genomic rearrangements could generate undesirable novel strains and variants of recombinant viruses used for vectored vaccines; more genome stability would be advantageous to maintain homogeneity of a master seed. Toward this end, Ball (1987) has begun examining intramolecular recombination mechanisms that might be manipulated to control the genome stability of vaccine candidate virus strains.

Topographical similarities and conserved sequences in the DNA of different poxviruses suggest that they evolved from a common ancestor (Drillien *et al.*, 1987). All temperature-sensitive mutants of vaccinia virus map to the central region of the genome, indicating the location of most genes essential for productive infection (Thompson and Condit, 1986). Hypervariability of the ITRs suggests that this region contains most nonessential genes (nonessential at least for cell culture infection). Terminal region mutability seems likely to be the key to virus adaptation. In this regard, several viable terminal region deletion mutants of vaccinia virus have been isolated for possible use as vectors (Perkus *et al.*, 1986; Panicali *et al.*, 1981; Moss *et al.*, 1981). Although DNA structural analysis has been performed on attenuated derivatives of Lister strain virus (Sugimoto *et al.*, 1985), the efficacy and safety of such strains for vectored viral vaccines remain to be determined.

D. Poxvirus Gene Expression

Poxvirus DNA is not infectious nor transcribed by cellular RNA polymerase. Both strands of DNA are transcribed via a distinctive RNA polymerase and other RNA-processing enzymes (for capping, polyadenylation, etc.); genes are spaced tightly along the poxvirus genome. Transcripts can be classified into three temporal classes: early, late, and early + late, relative to viral DNA replication (Moss, 1985). Annealing of RNAs from opposite DNA strands occurs very late in infection.

Leader sequences (30–100 bases) of vaccinia virus and early mRNAs appear to be different from each other and from prokaryotic eukaryotic mRNA leaders. Early mRNAs are of relatively uniform size, but late mRNAs; many for abundant proteins, vary in size. For example, the mRNAs for cowpox virus A-type inclusion protein and vaccinia virus structural 11-kD protein (both late proteins) have variable (5–35 bases) A-rich leader sequences (Bertholet *et al.*, 1987; Schwer *et al.*, 1987; Patel and Pickup, 1987). Evidence shows that the variable-sized A-rich leaders result from an inefficient RNA polymerase transcript initiation called "stuttering" (D. Pickup, personal communcation). Late mRNAs contain conserved UAAAUG sequences at the translation start codon that represent a conserved transcriptional processing site (Weir and Moss, 1987a; Hanggi *et al.*, 1986; Bertholet *et al.*, 1986). Slight deviations from the conserved sequences markedly diminish translation. The efficiency of translating individual late RNAs within an RNA group that shows varied 5′ ends are yet unresolved.

No genomic template has been found for the 25- to 100-base poly(A) tail on poxvirus mRNAs, but the signla for terminating transcripts at the 3′ end of poxvirus open reading frames appears in the genome as unevenly spaced tandem TTTTTNT sets (Rohrmann *et al.*, 1986; Yuen and Moss, 1986; Upton *et al.*, 1987). Functionally, it appears that viral mRNA capping enzyme plays a major role in terminating early transcripts by recognizing the UUUUUNU signal at the RNA 3′ end (B. Moss, personal communication).

Poxvirus RNA polymerase operates in concert with *cis*-acting promoters (AT-rich sequences immediately upstream of coding sequences). These promoters are distinctive from prokaryotic and eukaryotic promoters. Consensus TATA and AATA promoter sequences, separated by about 25 bp, have been identified for several early and late vaccinia virus genes (Pluciennicak *et al.*, 1985). Minimal promoter units of 15 to 100 bases, upstream of early gene transcription start sites and putative processing sites, have been resolved (Coupar *et al.*, 1987; Wier and

Moss, 1987a,b; Bertholet *et al.*, 1986; Cochran *et al.*, 1985). Interaction between poxvirus RNA polymerase and various promoters results in differential regulated production of mRNAs (Weinrich and Hruby, 1987). This, in turn, regulates the kinetics of protein synthesis. Regulation appears even more complex. For example, production and expression of the vaccinia virus TK, an early allosteric enzyme, may involve (1) feedback inhibition by TK metabolic products, (2) post-translational repression of TK synthesis, and/or (3) shutoff of TK mRNA synthesis by late transcription (Hruby, 1985; Hruby and Ball, 1981a,b; Franke *et al.*, 1985a; Bertholet *et al.*, 1987).

The influence of poxviruses on post-translational modifications of proteins, such as by phosphorylation and glycosylation, are unclear; in the case of simpler viruses, such modifications generally have been shown to be due to host cell enzymatic activities, but with poxviruses this remains to be explained. Some poxvirus proteins are post-translational cleavage products of precursor proteins, but again, whether the proteases carrying out such cleavages are of a host or viral source is not resolved. When proteins expressed by poxvirus vectors have been compared with native proteins, they usually have been found to be processed, transported through the cell, and secreted authentically. For example, influenza virus hemagglutinin, vesicular stomatitis virus glycoprotein, and murine leukemia virus envelope glycoprotein, when expressed by vaccinia recombinants, have been shown to be processed authentically and to migrate to apical or basal plasma membranes of host cells exactly like analogous native proteins (Stephens and Compans, 1986; Stephens *et al.*, 1986; McQueen *et al.*, 1986). An exception is represented by *Plasmodium falciparum* circumsporozoite protein, which when expressed by a vaccine virus vector was processed into two proteins (Smith *et al.*, 1984c). Further, a *Plasmodium* surface protein, when expressed via a vaccinia vector, was secreted from the host cell and thereby was poorly immunogenic; this was corrected by appending coding sequences for the transmembrane anchor domain of IgG to the *Plasmodium* gene (Langford *et al.*, 1986). Human immunodeficiency virus type 1 (HIV-1) envelope protein gp160, expressed via a vaccinia vector, was processed abnormally in HeLa and monkey kidney cell cultures, but was cleaved and glycosylated faithfully when expressed in a lymphocyte cell line (Chakrabarti *et al.*, 1986). Faithful processing of expressed flavivirus proteins also appears problematic (Deubel *et al.*, 1988).

E. Poxvirus Phenotypic Markers

The genetic nature of poxvirus phenotypic variation was first described by Fenner and his colleagues, who first demonstrated the existence of genetic linkage groups in animal viruses (Fenner, 1970, 1979, 1985a). Differentiation of poxvirus phenotypes has depended on detecting differences in many characteristics of virus growth and effects of virus growth in experimental animals, embryonating eggs, and cell cultures. For example, phenotypes have been described in terms of (1) variations in lesions produced by infection in the chorioallantoic membrane (CAM) of embryonating chicken eggs, in the skin of experimentally inoculated animals, and in cells in culture; (2) variations in the degree of virus dissemination and pathogenicity in experimentally inoculated animals; (3) variations in the amount of hemagglutinin and other antigens produced during infection in eggs and cell cultures; (4) variations in virus growth in eggs and cell cultures at restrictive temperatures; and (5) variations in host range in animals and cell cultures.

From the early 1900s, reactogenicity in rabbit skin and in the CAM of embryonating eggs had been regarded as the most reliable indicator of vaccinia vaccine safety and homogeneity. Vaccine stocks producing variable pock size or hemorrhagic pocks on CAMS or ulcerated lesions in rabbit skin were excluded from human use. As smallpox vaccination efforts intensified, exclusions were also based upon reported rates of adverse reactions in human recipients, such as severe local reactogenicity, high frequency of fever, etc. In the 1960s, as the WHO Smallpox Eradication Programme reached its peak, further efforts were made to correlate field reports of adverse reactions with laboratory tests. Still, most laboratory tests measured characteristics of virus replication in eggs and animals (Marennikova *et al.*, 1969; Koller and Zsidai, 1973; Marennikova, 1973). For example, one attempt to grade vaccines involved virus titration by intranasal inoculation of suckling mice; virulence for mice correlated reasonably well with clinical observations of fever and acute illness in vaccinated children (Krag-Andersen, 1969; Polak, 1973). None of these tests, however, could predict the frequency of the most severe, rare complications of vaccination, such as encephalitis. Even today, we are unable to make such predictions on the basis of practical laboratory tests.

Neuro-adapted virus strains (called "neurovaccinia" strains) have been studied extensively in experimental animals, but no study has yet led to a model of the human neurological disease caused rarely by vaccination (Hashizume *et al.*, 1973; Morita *et al.*, 1977; Briody, 1959). If a model were available, it would be used now to evaluate attenuated mutants of vaccinia virus as well as vectored vaccine candidates per se (Morita *et al.*, 1987; Neff, 1985; Hashizume *et al.*, 1985; Mayr *et al.*, 1978).

Today, evaluation of vaccinia virus phenotypic traits, such as neuro-

lence, is being approached by genetic analysis. Genes controlling
on structural and functional characteristics are being mapped, in-
ing, for example, vaccinia and cowpox virus genes involved in CAM
morphology and cell culture host range. The product of one vacci-
irus gene, a 29-kD protein, appears to be required for replication in
an cell cultures (Gillard *et al.*, 1986). Hemorrhagic CAM pock
acteristics, long associated with virulence, appear associated with
bition of blood coagulation, in part caused by a gene for a 38-kD
ein, which resembles mammalian plasma serine protease inhibi-
such as antithrombin-III (Pickup *et al.*, 1986). Other genes of
iruses encode proteins similar in amino acid sequence to host
eins. For example, a gene for a protein similar to mammalian
ermal growth factor (EGF) and transforming growth factor
F-α), which are cofactors in wound healing, has been identified
ardzik *et al.*, 1985; Stroobant *et al.*, 1985; Porter and Archard, 1987;
ng *et al.*, 1987). Genes encoding proteins similar to human and
n cell TK gene products, which affect general cellular replicat-
activity, have been identified (Bradshaw and Deininger, 1984;
h and Engler, 1984; Weir and Moss, 1983; Hruby *et al.*, 1983; Es-
to and Knight, 1984; Boyle *et al.*, 1987). It is hoped that complete
lyses of poxvirus genes and their products will help to characterize
is strains most suitable for substrates for producing vectored vac-
s.

t least 20 vaccinia virus genes have been analyzed, including the
e for the viral DNA polymerase, which resembles the polymerase
es of herpesviruses and adenoviruses (Earl and Moss, 1987;
leoch *et al.*, 1987; Earl *et al.*, 1986), and genes for subunits of the
l RNA polymerase, which resemble RNA polymerase genes of
st, *Escherichia coli*, and *Drosophila* (Broyles and Moss, 1986).
es for other vaccinia virion proteins, such as the 37-kD major
elope protein (Hirt *et al.*, 1986), the abundant type A inclusion
y protein (Patel *et al.*, 1986), and the hemagglutinin protein
da, 1986a), have also been analyzed. Relevant to vector virus
struction, deletion of the TK gene diminished vaccinia virus viru-
e in mice, rabbits, and chimpanzees (Buller *et al.*, 1985). The
etic basis of virulence is also being studied by constructing recom-
nts between vaccinia and ectromelia (mousepox) viruses, which
er in their CAM pock characteristics and pathogenic properties
rabbits and mice (Chernos *et al.*, 1985). None of these studies has
ught us to the point of being able to predict vaccinia vaccine char-
eristics or side effects, but they illustrate a rational approach to
problem.

F. Immune Response to Poxvirus Infection and Vaccination

Under natural conditions, poxviruses are transmitted by the respiratory route and by contact (some are also transmitted by arthropods), and infection is initiated in epithelial cells at the site of virus entry. The same occurs following poxvirus vaccination. The progression of infection is influenced by many factors, such as the virulence of the virus (substantial in the case of smallpox virus infection, minimal in the case of vaccinia virus vaccination), other biologic characteristics of the virus (e.g., tropism, replicating requirements), and innate determinants of the host response (specific and nonspecific cell-mediated immune responses, humoral immune response, age, hormonal effects, and action of interferons). Specific immunity is the principal mechanism for developing resistance against poxviruses; recovery from infection depends in large part upon the development of a cell-mediated immune response to viral proteins.

At sites of infection, virus particles are taken up first by macrophages, where in nonimmune hosts virus replication occurs (replication is abortive in immune hosts). Viral proteins produced via this infection are subsequently presented to induce specific T- and B-cell reactivities. Concomitantly, early NK-cell and cytotoxic T-cell activities are initiated. The cell-mediated immune response continues in infected epidermal (*stratum spinosum epidermidis*) and subcutaneous tissues, as well as other sites of virus replication *in vivo*. In the case of pathogenic poxvirus infections, the race between the virus and the host immune response may be settled in favor of either, but, in the case of vaccination, the race is settled quickly in favor of the host.

The cell-mediated immune response occurs via the production by T cells and specifically activated macrophages of a vast array of soluble mediators that affect virus, infected cells, and the local environment of the infection site. Eventually, these mediators even destroy virus within the pustules of pathogenic infections and the pustule that forms at the site of vaccination. In pathogenic poxvirus infections and in vaccinated individuals, the B-cell response generates nonneutralizing and neutralizing antibodies at about the time dermal vesicles begin to form and resolve into pustules. All of these host response events would be expected to be the same whether or not the infecting poxvirus expressed heterologous proteins. That is, it is most likely that vectored poxviruses will behave generally like their parents. However, similarities and differences must be determined directly with each vectored virus as it is advanced toward vaccine candidacy.

JOSEPH J. ESPOSITO AND FREDERICK A. MURPHY

IV. Poxvirus Vector Construction and Applications

A. Gene Transfer by Marker Rescue

h smaller viruses, genetic recombination is manipulated in the
ıtory via directed DNA ligation, but because of the very large
f the poxvirus genome, the method for inserting foreign genes
es site-directed homologous DNA recombination between the
virus genome and the heterologous insert. This method works
ıecause genetic recombination occurs readily in poxvirus infec-
in mixed poxvirus infections up to 50% of progeny are recombi-
. The historical development of infectious recombinant vectored
ıia virus and a summary of the gene expression method by thymi-
ıinase insertional inactivation are depicted in Figs. 1 and 2.
ıetic recombination was first studied in poxviruses under the term
genetic reactivation." This was done by coinoculation of two inacti-
poxviruses into animals, embryonating eggs, or cell cultures
ıer, 1970, 1979). Specifically, this phenomenon involves rescue of a
rus inactivated by heat or urea treatment (protein inactivation)
homologous or heterologous poxvirus inactivated by ultraviolet
(DNA inactivation). Coinoculation of protein-inactivated virus
an infectious preparation of virus of the same genus produces
nbinants with traits of both parents; such coinoculations involving
ologous poxvirus genera effect reactivation of the protein-
ivated virus but produce no recombinants. Reactivation occurs
ly because of cross-reactivity of poxvirus core-uncoating enzymes
ey and Cooper, 1987) and complementation between poxvirus
scription and gene regulation systems. For example, the fowlpox
TK gene, with its own promoter, has been expressed after inser-
into a TK vaccinia virus (Boyle and Coupar, 1986). Similarly,
s virus G and N coding sequences have been expressed under
ol of two different vaccinia virus promoters after insertion into
on poxvirus (Esposito *et al.*, 1988; W. Bellini, personal commu-
ion).

tanding classic studies on "nongenetic reactivat on," Sam and
bell (1981) reported the recovery of rabbitpox virus when its DNA
ransfected into cells that had been infected with ectromelia virus.
importantly, when they transfected DNA restriction fragments of
abbitpox strain of vaccinia virus into cells already infected with a
otypically different vaccinia virus, recombinants with rabbitpox
vaccinia virus phenotypic markers were recovered. Although gene
tions and deletions via targeted recombination had been used

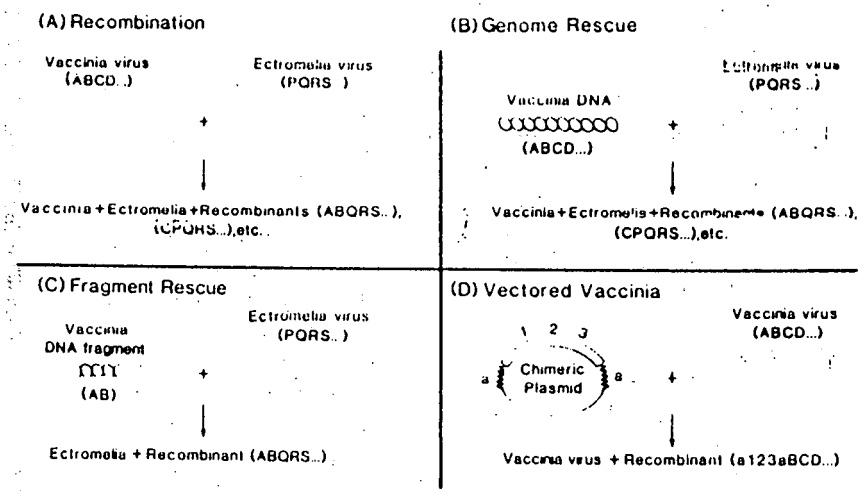

FIG. 1. Steps in the historical development of the use of vaccinia virus as an expression vector for heterologous DNA sequences. (A) Demonstration of recombination between vaccinia and ectromelia viruses by coinfecting cells with both viruses producing vaccinia, ectromelia, and recombinant viruses (Fenner 1970, 1979). (B) Rescue of vaccinia virus genome in cells transfected with intact vaccinia virus DNA and infected with ectromelia virus or temperature-sensitive mutants of vaccinia virus to produce vaccinia, ectromelia, and recombinant viruses (Sam and Dumbell, 1981). Genome rescue is similar to nongenetic reactivation in which cells are coinfected with heat-inactivated poxvirus and live poxvirus of homologous genus (Fenner 1970, 1979). (C) Marker rescue of fragment of vaccinia virus DNA by homologous DNA recombination in cells transfected with vaccinia virus fragment (marker DNA) and infected with marker virus (Sam and Dumbell, 1981; Weir *et al.*, 1982; Nakano *et al.*, 1982; Condit *et al.*, 1983). (D) Targeted insertion of heterologous coding sequences into vaccinia virus genome by homologous DNA recombination. Chimeric plasmid DNA that contains vaccinia virus DNA flanking the heterologous DNA is transfected into cells already infected with vaccinia virus (Blackett *et al.*, 1982; Panicali and Paoletti, 1982). Chimeric plasmid: a, vaccinia virus DNA flanking sequences; 1, vaccinia virus regulatory elements (promoter, RNA start); 2, heterologous DNA coding sequences; 3, vaccinia virus transcript termination elements. [From Fenner (1985a); redrawn with permission.]

arlier in prokaryotic systems (bacterial "homogenotization" and the
.e; Miller, 1972) and other eukaryotic systems, the experiments of
m and Dumbell marked the first demonstration of marker rescue for
oxviruses. About the same time, Post and Roizman (1981) described a
neralized method of selectable marker rescue for inserting or delet-
genes at specific loci in eukaryotic cells or viruses. They produced a
e deletion in a TK herpesvirus mutant by interrupting the func-
ing gene with TK sequences.

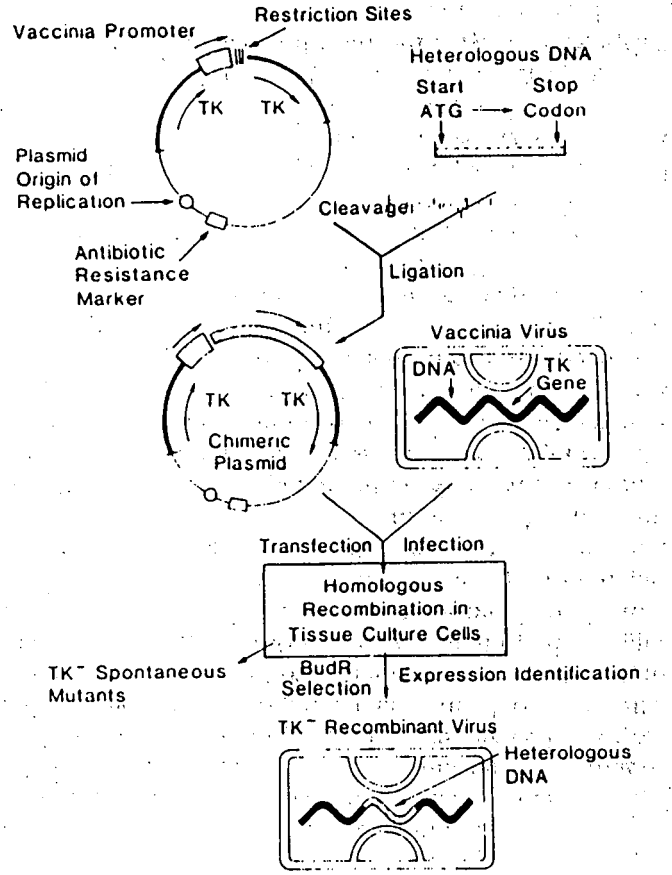

FIG. 2. Insertion of heterologous DNA coding sequences into vaccinia virus by insertional inactivation of the virus thymidine kinase (TK) gene. A chimeric plasmid is constructed in such a way that the coding sequences are inserted downstream of a vaccinia virus transcript start site that follows vaccinia virus promoter sequences; this cassette interrupts coding sequences for the vaccinia virus TK gene. Cells already infected with vaccinia virus are transfected with the chimeric plasmid before the peak of viral DNA replication. The infection is then allowed to proceed to completion. Three types of progeny virus are produced: wild-type TK⁺, spontaneous TK⁻ mutants, and TK⁻ recombinants. TK⁻ viruses are isolated by plaque purification using TK⁻ cells (mouse L, human 143, etc.) that are maintained in overlay medium containing 5-bromo-2′-deoxyuridine or trifluorothymidine. Recombinants are then differentiated from spontaneous mutants by hybridization and immunological methods using cells infected with virus from individual virus plaques (Mackett *et al.*, 1985a).

Separately confirming and extending the concept of poxvirus DNA fragment rescue, Condit *et al.* (1983), Nakano *et al.* (1982), and Weir *et al.* (1982) identified physical loci of *ts* mutations, reconstituted a vaccinia deletion mutant, and mapped the vaccinia TK locus. These studies led immediately to the development, by Panicali and Paoletti (1982) and Mackett *et al.* (1982), of methods for inserting heterologous coding sequences into vaccinia DNA, thereby producing the first infectious vectored vaccinia virus recombinants. In the studies of Mackett *et al.* (1982), the selectable system was based on interrupting the vaccinia TK gene; in these studies it was first noted that distinctive *cis*-acting vaccinia virus promoters were required for regulating foreign gene expression. This approach has been developed into a detailed protocol for selecting recombinants via insertional inactivation of the vaccinia TK locus, followed by plaque purifying the recombinants in TK⁻ cells under medium containing bromodeoxyuridine (Mackett *et al.*, 1985a).

The original methods for producing vectored vaccinia viruses involved targeted recombination between vaccinia virus genomic DNA and chimeric plasmids that contained a specific vaccinia DNA segment into which a heterologous DNA segment had been inserted. Briefly, such chimeric plasmids are constructed so that the flanking DNA correctly provides promoter and transcript leader sequences upstream of the heterologous DNA. All of the sequences must be in the proper reading frame, with the stop codon appropriately followed by a poxvirus transcript terminator. The poxvirus portions of the insert can be a contiguous part of the flanking DNA or include poxvirus regulatory elements translocated by genetic engineering from distal regions of the genome. The chimeric plasmid usually is transfected into cell cultures just after infection by the virus that is to serve as the vector. After a complete cycle of virus replication, recombinants are identified by differential plaque selection procedures using hybridization, immunofluorescence, or other tests. Selection of recombinants also has been accomplished by use of a restrictive temperature to rescue vectored *ts* mutants (Kieny *et al.*, 1984) and by use of coexpression of bacterial β-galactosidase (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986), neomycin phosphotransferase (Franke *et al.*, 1985b; Beaud *et al.*, 1987), or herpesvirus TK (Panicali and Paoletti, 1982; Mackett *et al.*, 1982; Moss *et al.*, 1986). A novel system has been described in which cells are coinfected with two vaccinia-vectored recombinants, one expressing T7 phage RNA polymerase and the second containing the desired heterologous gene fused between a T7 strong promoter and transcript terminator (Fuerst *et al.*, 1987). Vaccinia genome loci, such as those for hemagglutinin protein (Shida, 1986a,b), rifampicin resistance (Bal-

drick and Moss, 1987; Tartaglia *et al.*, 1986), and isatin-β-thiosemicarbizone dependents (Fathi *et al.*, 1986), have also been used to select recombinants.

B. Applications of Vaccinia Virus Vectoring in Vaccine Development

During the past few years, many genes coding for important proteins of viruses and other microorganisms have been inserted into vaccinia virus. Such recombinants have been used in basic studies of gene expression, transcription, protein processing, transport, secretion, etc. They have also been used in immunological studies, particularly for the generation of target cells expressing epitopes on their surfaces for use as cell-mediated immune reaction targets (Walker *et al.*, 1987), and, most importantly, in the development of infectious vectored vaccines. Several reviews and symposia have dealt with the application of vaccinia virus vectoring to vaccine development, some including partial listings of experimental products (Piccini and Paoletti, 1988; Moss and Flexner, 1987; Quinnan, 1985; Chanock *et al.*, 1988) Table III represents an overview of experimental products known to the authors. The listing illustrates the diversity of experimental vaccine development but is not comprehensive because many recombinants discussed informally have not been reported in the literature. In addition, a few examples of vectored vaccinia virus experimental vaccines are described below to illustrate particular advantages and disadvantages of the approach.

1. Vaccinia : Rabies Vaccines

The surface spike glycoprotein (G) gene of the ERA strain of rabies virus was expressed after insertion into the TK region of Copenhagen vaccinia virus (Wiktor *et al.*, 1984, 1988; Kieny *et al.*,1984). Mice, rabbits, skunks, raccoons, foxes, and cattle inoculated intradermally (ID) with this recombinant produced neutralizing antibodies and were protected (except cattle, not challenged; Koprowski *et al.*, 1987) against an otherwise lethal rabies virus challenge (Rupprecht *et al*, 1986; Blancou *et al.*, 1986; Wiktor *et al.*, 1988). Raccoons and foxes, but not dogs or skunks, were fully protected via the oral (PO) vaccination route. Lymphocytes from vaccinated mice were found to be primed for a secondary specific anti-rabies cytotoxic T-lymphocyte response. Because the Copenhagen strain, when used before the 1960s as a smallpox vaccine, had been associated with a high incidence of encephalitic complications (Polak, 1973; Krag-Andersen, 1969), six recombinants of the less

TABLE III

Vaccinia Virus Vectored Vaccine Candidates

Disease	Etiologic agent gene product	Vaccine application	Reference
Human			
Herpes genital disease	Herpes simplex virus types 1 and 2 gB, gD, gG	Universal vaccine	Sullivan and Smith (1987); Cremer *et al.* (1985); Paoletti *et al.* (1984)
Infectious mononucleosis	Epstein-Barr virus gp340	Universal vaccine	Mackett and Arrand (1985)
Cytomegalovirus disease	Human cytomegalovirus gB	Universal vaccine	Cranage *et al.* (1986)
Hepatitis B	Hepatitis B virus S, MS, LS, Core	Vaccine for high-risk groups in developed countries, universal vaccine for developing countries	Cheng and Moss (1987); Clarke *et al.* (1985)
AIDS	Human immunodeficiency virus gp 160, gp 120, gp41, gag, 3'ORF	Vaccine for high-risk groups, perhaps vaccine for highly endemic areas	Chakrabarti *et al.* (1986); Zarling *et al.* (1987); Zagury *et al.* (1987)
Respiratory syncytial disease	Respiratory syncytial virus G, F, N	Universal vaccine	Olmsted *et al.* (1985); King *et al.* (1987)
Parainfluenza	Human parainfluenza 3 F, HN	Universal vaccine	Spriggs *et al.* (1987)
Influenza	Influenza virus PB1, PB2, PA, HA, NA, NS1, NS2, NP, M1, M2	Universal vaccine, and vaccine for high-risk groups	Smith *et al.* (1987); M. Shaw, personal communication
Rotavirus gastroenteritis	Rotavirus SA-11 VP7	Universal vaccine for developing countries, vaccine for high-risk groups in developed countries	Andrew *et al.* (1987)
Rabies	Rabies virus G, N	Preexposure vaccine for high-risk groups, universal vaccine in some endemic areas	Esposito *et al.* (1987, 1988) and unpublished; Wiktor *et al.* (1986)

(*continued*)

TABLE III (*Continued*)

Disease	Etiologic agent gene product	Vaccine application	Reference
Lassa fever	Lassa fever virus G1, G2, N	Universal vaccine for endemic areas	Auperin *et al.* (1987, 1988) and unpublished; Clegg and Lloyd (1987)
Hemorrhagic fever with renal syndrome	Hantaan virus G1, G2, N	Universal vaccine for endemic areas	Pensiero *et al.* (1988)
Argentine hemorrhagic fever	Junin virus G1, G2, N	Universal vaccine for endemic areas	
Dengue fever	Dengue virus C, NS1, prM, M, E	Universal vaccine for endemic areas	Zhao *et al.* (1987); Deubel *et al.* (1988)
Malaria	*Plasmodium falciparum* and others CSP, merozoite S, RESA	Universal vaccine for endemic areas	Langford *et al.* (1988); Vijaya *et al.* (1988); Smith *et al.* (1986)
Tumor immunotherapy	Human melanoma-associated p97	Universal vaccine for high-risk groups	McKenzie *et al.* (1988); Estin *et al.* (1988)
Veterinary			
Rift Valley fever	Rift Valley fever virus G1, G2	Vaccine for sheep in endemic areas of Africa, and vaccine for stockpiling in case of introduction into other continents	Collett *et al.* (1987)
Foot-and-mouth disease	Foot-and-mouth disease viruses VP1	Vaccine for cattle in endemic areas, and vaccine for stockpiling in case of introduction into nonendemic countries	Clarke *et al.* (1988)
Rabies	Rabies virus G, N	Preexposure vaccine for cattle, dogs, and wildlife species in endemic areas	Esposito *et al.* (1987, 1988) and unpublished; Wiktor *et al.* (1988)
[illegible] encephalitis	virus capsid, E1-3, 6 kD	[illegible]	Kinney *et al.* (1989)
Vesicular stomatitis	Vesicular stomatitis viruses G, N	Vaccine for cattle	Mackett *et al.* (1985b)
Pseudorabies	Pseudorabies virus gp50, gIII	Vaccine for swine and cattle	E. Jones and D. Panicali, personal communication
Transmissible gastroenteritis of swine	Transmissible gastroenteritis virus gp195	Vaccine for swine	Hu *et al.* (1985)
Swine influenza	Swine influenza virus H1	Vaccine for swine, cattle, poultry	Boyle *et al.* (1986)
Equine influenza	Equine influenza virus H3, H7, N7, N8	Vaccine for horses	B. Cordell, personal communication
Avian influenza	Avian influenza virus H5	Vaccine for poultry	De *et al.* (1988)
Avian infectious bronchitis	Infectious bronchitis virus gp180	Vaccine for poultry	Tomley *et al.* (1987)
Feline leukemia	Feline leukemia virus gp70	Vaccine for cats	Gilbert *et al.* (1987)
Bovine leukemia	Bovine leukemia virus glycoprotein	Vaccine for cattle	[a]
Avian sarcoma	Avian sarcoma virus Pr ASV-C env	Vaccine for poultry	[a]
Bluetongue	Bluetongue viruses 1-24 VP2	Vaccine for cattle and sheep	[a]
Murine cytomegalovirus infection	Murine cytomegalovirus p89	Model for human cytomegalovirus prophylaxis	Koszinowski *et al.* (1988)
Lymphocytic choriomeningitis	Lymphocytic choriomeningitis virus G1, G2, N	Model for cell-mediated immunity	[a]
Sindbis virus infection	Sindbis virus C, PE2, E1, E2	Model for immune prophylaxis	Rice *et al.* (1985)
Babesiosis	*Babesia* sp./surface proteins	Vaccine for cattle	[a]
Anaplasmosis	*Anaplasma marginale*/surface proteins	Vaccine for cattle	[a]

[a] Uncited.

virulent NYBH strain of vaccinia virus (Fenner, 1985a; Krag-Andersen, 1969) that carried the G coding sequences of the CVS strain of rabies virus were produced in a collaborative study between virologists at the Centers for Disease Control and the National Institute of Allergy and Infectious Diseases (Esposito *et al.*, 1987). The six recombinants allowed researchers to compare the expression efficiency of two different promoters, the widely used $P_{7.5}$ (early-late class) promoter and the P_{11} (late class) promoter that had been presumed to be able to regulate higher levels of protein synthesis because it controls production of an abundant structural protein of vaccinia virus. Each of the six recombinants faithfully expressed rabies virus G protein conformational forms GI and GII, and each directed proper transport of this protein to the surface of infected cells. Three of the P_{11}-type recombinants produced in infected cell cultures about 10-fold more G protein than did the $P_{7.5}$-type recombinants. One P_{11}-type recombinant with an altered mRNA leader sequence induced about 100-fold less G protein than those with usual leader sequences. Single ID tail scarification or footpad (FP) inoculation with these vectored viruses induced rabies virus-neutralizing antibodies and protected mice against an otherwise lethal intracranial (IC) or peripheral FP challenge (Table IV). One vaccine candidate inoculated intramuscularly (IM) into dogs protected all recipients against an otherwise lethal rabies virus challenge.

As mentioned above, CVS rabies virus G and N coding sequences have been expressed via raccoon poxvirus. One P_{11}-type and one $P_{7.5}$-type chimeric plasmid that was used for developing vectored NYBH viruses was used again in recombinations to produce vectored raccoon poxviruses because of strong homology of TK sequences observed (Esposito and Knight, 1985) between these two orthopoxviruses. Oral vaccination experiments with raccoon poxvirus:G protein recombinants in animals showed induction of significant rabies virus-neutralizing antibodies in 100% of raccoons, dogs, cotton rats, and bobcats and in 33% of skunks; to date dogs, raccoons, and cotton rats have been challenged and were fully protected (Esposito *et al.*, 1988).

The G sequences of rabies virus also have been expressed after insertion into fowlpox virus using two systems: (1) an abortive infection of mammalian cell cultures and (2) a productive infection of avian cell cultures (J. Taylor, personal communication). Neutralizing antibodies were detected after injecting this recombinant fowlpox virus into rabbits (ID), rats (ID), cats (subcutaneous, SQ), dogs (SQ), cattle (SQ, ID, and IM), and mice (FP). Vaccinated mice were shown to resist challenge. On the basis of these studies, this fowlpox:rabies vaccine was proposed for use when a vaccinia:rabies vaccine might be unacceptable.

TABLE IV

RABIES VIRUS-NEUTRALIZING ANTIBODIES AND RESISTANCE OF MICE TO CHALLENGE AFTER VACCINATION WITH SIX DIFFERENT NEW YORK BOARD OF HEALTH VACCINIA:RABIES GLYCOPROTEIN VACCINES[a]

Vaccine	Neutralizing antibody		Mortality after challenge[b]
	Median	(Range)	
Intradermal			
A	5,700	(1,400–7,000)	0/11
B	5,700	(1,400–7,000)	0/12
C	1,600	(900–7,000)	0/7
D	2,400	(1,400–6,000)	0/9
E	3,125	(1,400–7,000)	0/11
F	6,200	(1,400–7,000)	0/9
Vaccinia control	5		11/11
Footpad			
A	1,400	(1,100–1,400)	0/12
B	1,400	(625–1,400)	0/12
C	280	(40–1,800)	0/10
D	1,000	(280–5,100)	0/9
E	1,200	(56–6,300)	0/10
F	1,400	(625–3,125)	0/11
Vaccinia control	5		10/10

[a] All vaccines used the NYBH strain of vaccinia virus and a recombinant insert derived from the G gene of the CVS strain of rabies virus. In vaccines A–D, the rabies virus gene was expressed under control of the vaccinia promoter P_{11}; A and B were fusion protein versions, C had an altered mRNA leader, and D mimicked the promoter and leader of the naturally occurring 11-kD vaccinia gene. In vaccines E and F, the rabies gene was expressed under the control of the vaccinia promoter $P_{7.5}$; they differed in sequences flanking the translation start codon.

Four-week-old female A/J mice were inoculated with 10^7 PFU of virus. Mice were bled 4 weeks postinoculation, tested for antibody, and challenged 2 weeks later by footpad inoculation of 10 MLD_{50} of street rabies virus (CDC, Mexico dog strain 2699) (Esposito *et al.*, 1987).

Poxvirus-vectored rabies vaccines could play a significant role in animal and human rabies control programs, but this must be evaluated in regard to risk:benefit, separately, in each of several settings:

1. *Human vaccine in developed countries.* Poxvirus recombinants would have to be compared with presently available, very potent, very safe, inactivated cell culture-derived vaccines. These vaccines are

largely used for postexposure prophylaxis where speed of the immune response, not cost, is most important. In fact, the major disadvantage of presently available vaccines is their very high cost, which surely would be reduced if poxvirus recombinant products were to meet current standards for safety, efficacy, and speed of antibody induction.

2. *Human vaccine in developing countries.* Poxvirus recombinants would have to be evaluated as above in regard to efficacy, safety, and speed of antibody induction, but against a much poorer standard—the standard set by present rabies vaccines that are derived from neural tissues of animals. The prospect of this is not clear, but the probable low cost and practicality of local production of vectored poxvirus vaccines suggest that further study is worthwhile.

3. *Pet animal vaccines in developed and developing countries.* Poxvirus recombinants would have to be compared with presently available potent and safe attenuated-live and inactivated rabies vaccines. These vaccines are used only for preexposure prophylaxis, where efficacy, safety, and cost are important. Safety considerations would have to include a sessment of risks of transmitting vectored virus from animals to humans. Because of this, the prospect of using poxviruses that cause abortive infection, such as fowlpox virus, appears to warrant further study.

4. *Livestock animals in developed and developing countries.* Poxvirus recombinants would have to be evaluated against a tradition for very little vaccine usage, even in areas where losses due to rabies are substantial. Because poxvirus-vectored vaccines would have a low cost and would be easily administered, this is an exciting prospect.

5. *Wildlife species in developed countries.* Poxvirus recombinants would have to be compared with attenuated live-virus vaccines now being used in Canada and a few European countries to immunize some wildlife species via oral bait-delivered vaccine. For wildlife species in which these attenuated-live rabies virus vaccines are not immunogenic by the oral route, vectored vaccines represent a novel approach to a most difficult problem. The possibility of using other viruses as infectious vectors, such as raccoon poxvirus (Esposito *et al.*, 1988) or canine adenovirus (infectious canine hepatitis virus), could add the extra element of host range specificity to such vaccines. All of these approaches offer exciting prospects.

2. *Vaccinia : Lassa Fever Vaccine*

Vaccinia virus recombinants expressing Lassa virus (Josiah strain glycoproteins (G1 and G2 via cleavage of polyprotein GPC) (Auperin *et al.*, 1987, 1988) or the nucleocapsid protein (N) (Clegg and Lloyd, 1987; D. Auperin, personal communication) have been produced and are being evaluated for use in a candidate human vaccine. When a NYBH vaccinia : Lassa virus GPC recombinant was inoculated intradermally into guinea pigs or rhesus monkeys, it evoked only low levels of nonneutralizing G-specific antibodies. However, all monkeys and most guinea pigs survived lethal challenge. At the time, when standard NYBH virus vaccinated control animals became sick and died, the GPC recombinant vaccinated animals had a brief low-grade viremia and transient fever. No significant protection of rhesus or cynomolgous monkeys was observed with a NYBH : Lassa (Josiah strain) N recombinant virus made at the Centers for Disease Control. The NYBH : N and a Lister vaccinia : Lassa (Nigeria strain) N recombinant, produced at the Public Health Laboratory Service at Porton Down, England, protected guinea pigs. The reasons for these contrasting results have not been resolved. The present results, taken together with those of other studies (J. McCormick, personal communication), have suggested that neutralizing antibodies are not critical in the recovery of inoculated animals or naturally infected humans from Lassa virus infection. Rather, growing evidence shows the importance of a cell-mediated immune response elicited against the virus proteins.

Coupling of two fundamental epidemiological observations makes the need for a human vaccine against Lassa fever most urgent; in West African countries more than 300,000 cases of the disease are estimated to occur annually, and every attempt to date to develop a vaccine by conventional approaches has failed. Inactivated virus vaccines have not been efficacious in damping viremia or in protecting experimental animals, and attempts to use a naturally attenuated variant, Mopeia virus, are stymied because of the inability to predict in animal models the possibility of rare adverse events, such as CNS invasion. In this context, the development of a usable vaccinia : Lassa virus vaccine represents an exciting prospect. In West Africa, this development must include concern for adverse effects of vaccinia virus use in persons immunosuppressed by HIV infection.

3. *Vaccinia : Venezuelan Equine Encephalitis Vaccine*

Vaccinia virus recombinants carrying the 26S polygene of Venezuelan equine encephalitis (VEE) virus (strains TC-83 or Trinidad donkey) have been produced at the Centers for Disease Control (Kinney *et al.*, 1989). When inoculated into BSC-40 monkey cell cultures, the recombinants faithfully expressed the native polyprotein, which was normally cleaved to form all of the virion structural proteins (map order: capsid, E3, E2, 6 kD, E1). Colony-bred NIH Swiss and inbred A/J and C3H strains of mice were inoculated with the recombinants intradermally (via tail scarification). Mice developed VEE virus-

neutralizing antibodies and were protected against an otherwise lethal intraperitoneal challenge with virulent VEE virus (Table V).

Lymphocytes isolated from animals inoculated with TC-83 or the recombinant vaccinia virus vaccine exhibited VEE-specific *in vitro* proliferative responses. Protection was demonstrated against each subtype of VEE. Protection against intranasal challenge, however, was only 10–20% effective. Vaccinated, challenged mice developed very high antibody titers, presumably due to subclinical systemic infection. The recombinants were as effective as the attenuated-live virus TC-83 vaccine in inducing cross-protection against challenge with subtypes of VEE virus isolated from outbreaks. These recombinants are now being developed as candidate vaccines.

The potential value of a vaccinia : VEE recombinant vaccine must be considered against needs as they occur in the face of epizootics/epidemics:

1. *Equine vaccine.* Epizootic VEE has been devastating to equine populations in Central and South America, in areas where agriculture is heavily dependent upon horses and mules. Increasing numbers of infected horses and mules in an outbreak amplify the presence of VEE virus, thereby furthering risks to human populations. The present attenuated live-virus vaccine (strain TC-83) is efficacious but suffers from cold-chain and other logistic problems. To prove its value, a vaccinia virus recombinant product will have to be evaluated in realistic settings in Central or South America; this is an exciting prospect.

2. *Human vaccine.* Laboratory workers and disease control field workers are at high risk of infection and disease; a vaccine is necessary to protect such persons who may contract the disease via insect bite, accidental inoculation, or aerosol. TC-83 vaccine, formulated for human use, appears generally efficacious, but does cause adverse effects in some recipients. An inactivated cell culture-derived vaccine (strain TC-84) is safe but not very potent. A vaccinia virus recombinant product would have to be evaluated against this background; this is an exciting prospect.

4. *Vaccinia : Influenza Vaccines*

a. Vaccinia : Avian Influenza (H5) Vaccine. Avian influenza H5 virus was selected at the Centers for Disease Control for use as a model system to evaluate the feasibility of using a vaccinia virus-vectored vaccine to interrupt rapidly moving epidemics/epizootics, such as those caused by human and avian influenza viruses. The hemagglutinin (HA) of avirulent A/Chicken/Scotland/59 virus was expressed using the

TABLE V

VENEZUELAN EQUINE ENCEPHALITIS (VEE) VIRUS ANTIBODIES AND RESISTANCE OF MICE TO CHALLENGE AFTER VACCINATION WITH NYBH VACCINIA VEE VACCINE

Vaccine	Antibody titer (reciprocal, mean)[a]			Mortality after challenge
	Neutralizing	HI	ELISA	
Vaccinia : VEE				
Prechallenge	1,180	43	2,100	
Postchallenge	55,000	890	86,000	0/8
Vaccinia control	<5	<5	<5	8/8
VEE TC-83				
Prechallenge	51,200	340	Not done	
Postchallenge	56,000	1,560	Not done	0/8

[a] Five-week-old mice were inoculated intradermally (tail scarification) with 10^4 PFU of vaccinia : VEE vaccine or intraperitoneally with 10^4 PFU of TC-83 attenuated-live virus vaccine. Mice were bled 19 days postinoculation, tested for antibodies, and challenged by intraperitoneal inoculation of 100 MLD_{50} of virulent VEE virus (Trinidad donkey strain). Fourteen days later, surviving mice were bled again and tested for antibodies.

NYBH vaccinia virus vector system (De *et al.*, 1988). In human 143 cells, the HA expressed via the NYBH vector was slightly larger by electrophoresis than the influenza virus authentic protein, possibly because of extra glycosylation, otherwise it appeared identical to the native HA. Vaccine was prepared from γ-irradiated vector virus-infected cells by homogenization with Freund's complete adjuvant. Adult chickens, immunized intramuscularly with this preparation, developed H5 influenza virus-neutralizing and hemagglutination-inhibiting (HI) antibodies and were protected against an otherwise lethal challenge with A/Chicken/Scotland/59 or A/Chicken/Pennsylvania/83 virulent influenza viruses (Table VI). Kuroda *et al.* (1986) had previously demonstrated the protective value of an H7 hemagglutinin protein produced in a baculovirus expression system. Taken together, the two experiments suggest the merit of vaccination, rather than slaughter and disinfection, in the face of avian influenza epizootics. By extension, the two experiments suggested the merit of considering similar vaccines for human influenza. It will now be of interest to express H5 and H7 proteins using a vaccine strain of fowlpox virus, thereby adding the advantages of an infectious vectored virus vaccine for poultry.

TABLE VI

AVIAN INFLUENZA ANTIBODIES AND RESISTANCE OF ADULT CHICKENS TO CHALLENGE AFTER VACCINATION WITH AN INACTIVATED VACCINIA : H5 INFLUENZA VIRUS (A/Chicken Scotland 59) HEMAGGLUTININ VACCINE[a]

Vaccine	Antibody titer (reciprocal, mean)		Mortality after challenge
	HI	Neutralization	
Vaccinia H5-HA			
Prechallenge	5	240	
Postchallenge	112	6,400	1/20
Vaccinia control	5	5	20/20

[a] Chickens were inoculated subcutaneously with NYBH vaccinia : H5 avian influenza virus hemagglutinin vaccine prepared in chick fibroblast cells. The recombinant vaccine contained $10^{8.7}$ PFU/ml; it contained 0.5 μg of H5-HA protein per dose. The vaccine was inactivated by γ-irradiation and homogenized in Freund's complete adjuvant. At 14 days after vaccination chickens were tested for antibodies and challenged with 10^6 PFU of virulent A/Chicken/Scotland/59 influenza virus. Serum specimens from surviving birds were tested 32 days after challenge.

b. Vaccinia : Influenza B Vaccine. When influenza B viruses are cultivated in embryonating chicken eggs or in cell cultures, antigenically distinct variants are differentially selected (Schild *et al.*, 1983). These variants differ by single amino acid substitutions in a key domain of the hemagglutinin. To decide which host system, eggs or cell cultures, is most suitable for producing the most efficacious vaccine, researchers at the Centers for Disease Control constructed two NYBH vaccinia virus recombinants, one containing the HA gene from B/England/222/83 virus grown in eggs and the other, the same gene from the same virus grown in MDCK cells (Rota *et al.*, 1987). The vaccinia virus recombinants were used to vaccinate groups of mice, which were then separately cross-challenged with B/England/222/82 virus grown in either host system. All mice were protected against challenge. When the sera of the challenged mice were used in cross-absorption tests, however, a much broader neutralizing antibody response was evoked by the vaccinia virus recombinant expressing the HA of the egg-derived virus. Because of uncontrollable mutations continuing during growth of influenza viruses in such experiments, the vaccinia virus recombinants were crucial for stabilizing the respective HA genes in DNA form. In parallel experiments, serum specimens from persons immunized with influenza B vaccine (derived from B/Singapore/222/79 strain virus, grown in eggs) were found to have comparable neutralization titers against influenza B virus grown in eggs or MDCK cells. These findings contribute to proof that the current influenza vaccine manufacturing process, using embryonating eggs, produces vaccines that are broadly efficacious against viruses circulating in nature. Of course, the very broad immunogenicity that is required of influenza vaccines to protect against the different viruses circulating in nature at any given time must be achieved by incorporating into vaccine multiple viruses and by changing these as viruses change in nature.

5. *Other Vectored Poxvirus Vaccine Candidates*

As illustrated in Table III and with the development of vectored fowlpox and raccoon poxvirus, many choices are available for initial trials (phase 1, 2, and 3 clinical trials in humans, closed and open field trials in livestock animal species) of vectored virus candidate vaccines. Yet no single candidate, for either human or animal use, can be easily identified as being above controversy. The choices for initial trials must be based on analyses of risk : benefit, cost : benefit, alternative product availability, public acceptability, environmental impact, etc. For some candidate vaccines, considerations may be based upon universal factors, but for others, including the examples given in the preceding

sections, needs are limited to selected populations in limited geographic areas and considerations are greatly affected by varying views of these needs. Candidate human vaccines against AIDS, respiratory syncytial virus disease, hepatitis B, dengue, and malaria, and candidate animal vaccines against rabies, vesicular stomatitis, and transmissible gastroenteritis of swine have received much attention. Each of these candidates raises complex questions, as does the concept of infectious vectored virus vaccines in general. As a result, there is a perceived inertia in moving from feasibility experiments in laboratory animal species to clinical and field trials. Although a vaccinia : HIV-1 env-protein vaccine candidate has been used experimentally in humans in Africa (Zagury *et al.*, 1987) and a vaccinia : rabies G is being field-tested in Belgium (WHO Veterinary Public Health Unit, personal communication), one can still ask: "Which should go first, in the context of which vaccine candidate can best meet an immediate need and at the same time best contribute to our understanding of infectious vectored virus vaccines in general?" Clearly, different investigators, research institutions, government agencies, and regulatory authorities will answer these questions differently, but perhaps in ways that overcome the inertia of the day.

V. Other Infectious Vectored Virus Vaccines

A. Vectored Herpesvirus Vaccines

No one virus or family of viruses identified has ideal characteristics for use as the substrate for all infectious vectored virus vaccines. Differences in the desired usage of vaccines are such that different vectors may be envisioned in different circumstances. Hence, the feasibility for using any virus with a large enough genome to carry heterologous genes must be considered. In this regard, the herpesviruses offer several unique advantages, and a few disadvantages.

In general, the herpesviruses evoke very strong cell-mediated immune responses, as well as strong and long-lasting humoral responses. Additionally, the viruses have rather narrowly specific host ranges, which might be considered advantageous in any live-virus vaccine. Particular herpesviruses are also associated with neurologic and neoplastic diseases, however, and all herpesviruses are associated with persistent infection and life-long carriage. These characteristics, unless further defined and absolutely controlled, would be considered unacceptable in any live-virus vaccine.

The biology, molecular biology, and immunobiology of the human and animal herpesviruses have been reviewed (Roizman, 1985; Roizman and Lopez, 1985; Lopez and Roizman, 1986). Herpesviruses contain linear double-stranded DNA (120–250 kbp), which varies in physical structure among genera and encodes 50–80 genes. Purified DNA is infectious. Virus replication takes place in the nucleus and virion maturation, via envelopment, takes place at the nuclear and plasma membranes of the infected cell. Replication requires cellular polymerase II for transcription and expression of viral proteins. Polypeptides arise in a cascade fashion, coordinately regulated and sequentially ordered. For example, immediate-early α-gene products are required for β-gene expression, the products of which in turn effect late γ-genes that code for virion structural proteins.

Methods for the insertion, deletion, and substitution of endogenous and exogenous DNA sequences into herpesviruses, particularly herpes simplex virus, via homologous recombination, have been reviewed (Jenkins and Roizman, 1986; Roizman and Jenkins, 1985; Roizman and Arsenakis, 1985a,b; Dix, 1987). Herpes simplex virus has been used successfully as a vector for expressing hepatitis B virus surface antigen (Shih *et al.*, 1984) and for Epstein Bar virus nuclear antigen 1 (Hummel *et al.*, 1986). An attenuated varicella virus strain (OKA), which has been licensed in some countries for use as a universal vaccine or special-use (in pediatric leukemias, etc.) vaccine, has been used for the expression of EB virus gp350/220 glycoprotein (Lowe *et al.*, 1987).

Unique prototype herpes simplex virus vectors have been developed (Meigner *et al.*, 1988). They consist of herpes simplex type 1 virus from which 15% of the genome has been deleted and into which genes for herpes simplex type 2 virus immunogenic proteins (gG, gD, gI) have been inserted. Because of the extent of the deletions, these viruses have genomic space for the insertion of more than 10 kbp of heterologous DNA. Safety and immunogenicity of these recombinants have been examined in experimental animals. These recombinants do not disseminate from the site of inoculation nor do they cause systemic infection. Neurovirulence has been eliminated; direct intracerebral inoculation of experimental animals does not appear to cause infection. Both recombinants have been shown to protect mice, guinea pigs, rabbits, and two species of primates against challenge with wild-type herpes simplex type 1 and 2 viruses. These recombinants could be configured as a human vectored vaccine against several viral diseases.

Although persistent, life-long infection is usually viewed as an undesirable characteristic in regard to use of herpesviruses as infectious

vectored vaccines, it can also be viewed as a unique advantage. The immune system of the person or animal receiving a herpesvirus-vectored vaccine would be continuously or intermittently (via recrudescence) stimulated. Although the genes responsible for the persistence of herpesviruses have not been identified, it is thought that they must be nonessential for basic virus replication, and therefore removable. Proponents of vectored herpesvirus vaccines have indicated that, at least for herpes simplex type 1 and 2 viruses, this is feasible (Roizman and Arsenakis, 1985a,b; Meignier *et al.*, 1988). The counterargument is that we may never know enough about the complex viral characteristics that lead to herpesvirus persistence to warrant the risk involved in vaccination.

Persistence and long-term immunostimulation would be a disadvantage in regard to vectored vaccine usage if one vaccination precluded the effectiveness of subsequent vaccinations. However, evidence shows that (1) individuals with preexisting high-titered antibody to herpes simplex type 1 virus do allow local replication of superinfecting virus and (2) individuals with a strong immune responsiveness to herpes simplex type 1 virus allow infection and replication of type 2 virus. So, it remains to be seen whether multiple vaccination regimens, using different vectored herpesviruses, would be efficacious.

Many attenuated-live virus vaccines for herpesvirus diseases are now being used in livestock, companion animals, and poultry. Each of these can be considered as a potential substrate for vectored vaccines for the host species. Because herpesviruses are such important pathogens of animals and poultry, very large numbers of doses of these attenuated-live virus vaccines are now used. Therefore, it might be anticipated that vectored vaccine products that protected against the homologous substrate virus as well as the agent of the heterologous insert would have a strong marketing base. The value of each vaccine virus chosen for use as a vector would have to be judged independently of its value as an attenuated-live virus vaccine, but it would be the combined homologous and heterologous immunogenicity that would determine vaccine usage. Because of the narrow host specificity of the animal and poultry herpesviruses, the overall environmental risk of use of infectious vectored herpesvirus vaccines would be expected to be limited, but this would have to be proven in closed and open field trials.

B. Vectored Adenovirus Vaccines

Like the herpesviruses, the adenoviruses have advantages and disadvantages in regard to their use as vectors for human and animal vaccines. Adenoviruses are capable of evoking good humoral and cell-mediated immune responses, and in natural respiratory tract infections they evoke good mucosal immune responses. The viruses have narrowly specific host ranges, usually considered an advantage for live-virus vaccines.

Since the 1960s, U.S. military personnel have been vaccinated with a live-virus vaccine to prevent acute respiratory disease, a particular problem in training facilities. Outbreaks of respiratory disease in such settings are most often caused by adenovirus types 3, 4, 7, 14, or 21. The vaccine consists of nonattenuated cell culture-propagated adenovirus types 4 and 7. The vaccine is administered orally via enterically coated capsules, which avoids respiratory tract infection and virus denaturation in the stomach. Infection of the intestinal tract is asymptomatic, but does result in effective immunity against the homologous virus types and lesser, but valuable, immunity against the other virus types that cause disease in these settings. Because of this successful experience, current proposals for the use of adenoviruses as vectors for human vaccines have started with the licensed vaccine viruses and with enterically coated capsules as a delivery system.

The biology, molecular biology, and immunobiology of the adenoviruses have been reviewed (Ginsberg, 1984; Doerfler, 1984). Forty-three human adenovirus types, organized into seven major groups, have been identified. The viruses replicate in the nucleus and are released by cytolysis. Their genome consists of one molecule (36 kbp) of double-stranded DNA with ITRs; the genome encodes 11–15 structural proteins and at least 15 nonstructural proteins. The small size of the adenovirus genome and virion packaging constraints limit the amount of heterologous DNA that may be inserted.

The feasibility of using adenoviruses as infectious vectored vaccines was first explored with human adenovirus type 5, which can be evaluated in a hamster model. Hepatitis B virus surface antigen (HBsAg) coding sequences under control of the adenovirus major late promoter (MLP) was inserted by homologous recombination into the genome of adenovirus, downstream of the promoter for the early E3 region (Morin *et al.*, 1987). This has been done with prototype adenovirus type 5, with an E3-deletion mutant, and with the adenovirus type 7 vaccine strain (Morin *et al.*, 1988). The insert was placed to immediately precede or replace coding sequences for the adenovirus 19-kD glycoprotein, a protein that is apparently unnecessary for viral replication in cell culture. When these recombinants were inoculated intranasally into hamsters, they evoked antibodies to both adenovirus and HBsAg. Other adenovirus recombinants have been developed: one adenovirus type 5:HBsAg recombinant when inoculated intravenously into chimpan-

zees produced partial protection against challenge (Ballay *et al.*, 1985; Levrero *et al.*, 1988).

In adenovirus infections, the 19-kD viral glycoprotein may mediate the host cellular immune response; the protein appears to affect the expression on infected cells of class I major histocompatibility antigens that are part of the immune recognition site. This phenomenon may significantly modulate infected cell evasion from immune surveillance (Severinsson *et al.*, 1986; Pettersson, 1984). Adenovirus types 4 and 7 vectors have been of value with an MLP : HBsAg cassette inserted near the right-end ITR, which has provided evaluation of expression of the 19-kD glycoprotein (B. Mason, personal communication).

The prospect for the use of vectored adenovirus vaccines in humans cannot be fully evaluated at this time. The prospect of having vaccines that could be delivered orally is exciting, but the limited heterologous gene carrying capacity of the viruses, their nuclear site of DNA replication, their mRNA splicing capability, their ability to cause transformation in cell cultures and tumors in animals (under highly artificial conditions; Graham, 1984), their ability to form functional hybrids with SV40 virus (Klessig, 1984), and their persistence in lymphoid tissues represent unanswered concerns.

The prospect for the use of vectored adenovirus vaccines in animals seems much more exciting. More than 50 adenoviruses of animals have been identified, some recognized as important pathogens, and some attenuated and used as live-virus vaccines. For example, the insertion of genes for protective epitopes of canine adenovirus type 2 into the attenuated live-virus vaccine strain of canine adenovirus type 1 (infectious canine hepatitis virus) represents a simple approach to a bivalent vaccine. A more far-reaching prospect would be the expression of rabies virus G and N coding sequences by an attenuated live-virus vaccine strain of canine adenovirus type 1. Such a recombinant vaccine with the infectiousness and transmissibility of the parent adenovirus vaccine strain could be used as an oral rabies vaccine for dogs and even for raccoons (also infectable by this adenovirus). Like the parent canine adenovirus vaccine strain, an adenovirus : rabies recombinant might be transmitted naturally from vaccinated animals to other susceptible contact animals, thereby achieving a greater herd immunity than otherwise possible.

VI. Conclusions

In the long term, ideas for better vaccine usage are dominated by the potential of genetically engineered vaccines. The "ideal vaccine" of

today is best represented by some of the pediatric attenuated live-virus vaccines. In fact, when we insist that the "ideal vaccine" mimic natural infection in ts influence upon the recipient, we set a very high standard, a stand ırc that so far has been most easily attained by attenuated live-virus vaccines. This standard will be difficult to match with any genetically engineered product, unless full attention is paid to cell-mediated and humoral immunogenicity as well as other host responses. Likewise, new vaccines must be evaluated in regard to their capability to evoke maternal immunity that is transferable to the fetus and newborn and their efficacy in the face of prior vaccination or maternal antibodies. New vaccines must also be evaluated appropriately in regard to their thermal stability, that is, their usefulness in areas where the "cold-chain" is not reliable. All in all, it seems clear that there are today too few "ideal vaccines," but it is still too early to judge how infectious vectored virus vaccines will contribute to an overall vaccine armamentarium.

Vectored virus vaccines should be considered model systems in the overall context of safe vaccine use in disease control. For example, small genetic changes in attenuated live-virus vaccine seed stocks can lead to the production of vaccine lots with unacceptable pathogenicity. For this reason, attenuated live-virus vaccines will always have to be proven safe on a lot-by-lot basis. For the vaccine producer, it may well be attractive to eliminate this problem by eliminating all need of working with mutable seed viruses. Recombinant DNA viruses carrying desired genes for heterologous immunogenic proteins would be genetically very stable. They would also allow the rapid production of large amounts of vaccine in a short time, as required in the face of epidemics and epizootics. In such settings, recombinant vaccines need not overburden safety and quality assurance testing, nor other aspects of the regulatory process.

A. Outlook for Infectious Vectored Virus Vaccines for Humans

Member viruses of the three families (Poxviridae, Herpesviridae, Adenoviridae), described here as having potential for use as infectious vectored virus vaccines, have characteristics that lead to a complex judgment as to the overall promise for the practical use of end products in the field. Feasibility seems clear even though, in most cases, questions of stability of recombinants and level of expression of inserted gene products have not been fully answered. Virus vectors now under development have the ability to carry enough heterologous DNA to code for single or multiple genes for immunogenic proteins. Vector

systems allow remarkably faithful transcription and translation from inserted genes, as well as proper post-translational processing and transport. Some researchers are optimistic that increased expression will be achieved by the use of better natural or synthetic promoters. Similarly, some are optimistic that increased immunogenicity may be achieved by novel linkages of genes for immunogenic proteins with those for lymphokines and other immunomodulators (Flexner *et al.*, 1988).

At this time, the major concern over the use of infectious vectored virus vaccines for humans is safety. In the face of a substantial rate of disease occurrence and conditions affecting immunocompetence (chemical immunosuppression, AIDS, malnutrition, etc.), the safety of all live-virus vaccines, including infectious vectored vaccines, must be assured. After a U.S. military recruit, who was unknowingly infected with HIV, developed disseminated vaccinia after routine vaccination (Redfield *et al.*, 1987), it was suggested that all research on recombinant vaccinia vaccines be discontinued. Halsey and Henderson (1987) retorted: ". . . it would be extremely unfortunate if routine immunizations were unnecessarily withheld because of over-interpretation of information and speculations [from one case of vaccination complication] . . . ." Further, they noted that available data indicated that several hundred HIV-seropositive military recruits must have received many different immunizations without ill effects before routine screening and exclusion of HIV-positive applicants.

Genetic manipulation of vectored viruses to minimize their pathogenicity is one major response to safety concerns. This approach is based upon understanding of the functions of the many viral genes that contribute to virulence characteristics, such as invasiveness, tropism, escape from nonspecific host defenses, escape from specific host defenses, etc. Although this is a very complex area of research, much progress is being made. However, progress made in the laboratory toward developing candidate vaccines with predictable and stable safety characteristics will only be worthwhile if it is complemented by progress in developing a reasonable public perspective of biotechnology and its products.

Public "informed consent" must involve public interest groups that are critical of biotechnological (genetic engineering) development. Such consent must achieve widespread awareness and agreement that these vaccines are needed and are safe. Certain public interest groups active in this subject area, such as the Foundation on Economic Trends in the United States and the Green Party in the Federal Republic of Germany, have taken the position that all genetic manipulation involves far greater risk than is generally realized and thus must be prohibited (American Society for Microbiology, 1987a). Such groups have taken legal action affecting experimentation per se, the evaluation of experimentation, and the regulation of experimentation and its products (American Society for Microbiology, 1987b). Other groups have sought to dissect the overall subject into its parts, so that separate judgments might be made. In this case, vaccines become quite appealing because of their unique cost-effective value for tackling difficult problems in developed and developing countries. At this point, it seems that the attitude of most scientists and government agencies in most countries, as reflected in extramural research funding and intramural research programs, is very supportive of developmental research but rather cautious in regard to clinical and field trials. In these same countries the attitude of the public is still not clear. In the opinion of the authors, biotechnologically produced products, including vaccines, can be made safe, and public confidence in the safety, efficacy, and value of these products can be gained by full disclosure and informative communication. Important positive elements in this process are contained in the proposal by the U.S. Office of Science and Technology for a "Coordinated Framework for Regulation of Biotechnology," (Federal Register, 1985).

B. Outlook for Infectious Vectored Virus Vaccines for Animals

From the first days of the genetic engineering revolution, some researchers believed that vaccines for animal diseases would be the proving ground for human vaccines. It was thought that funding would flow into this research field because demands for vaccine safety and efficacy are less stringent, licensing is simpler, and markets are larger. To some extent this has been the case; according to a survey done for the Center for Veterinary Medicine, U.S. Food and Drug Administration (American Society for Microbiology, 1986), approximately 100 firms have been pursuing at least 200 separate veterinary biotechnology projects in the United States. The largest number of these projects have been for disease management, i.e., diagnostics reagents and kits, therapeutics, and in the largest numbers, vaccines. Nevertheless, the premise that veterinary products represent the brightest area for biotechnology enterprise has partially collapsed, and many of the largest companies are shying away from the animal vaccine market. This disillusionment should be examined—perhaps promises did outdistance reality, but, in view of the most recent developments in the technology and its applica-

tion, there seems reason for optimism. The promise of vectored virus vaccines should be seen as a specific reason for optimism.

In several livestock industries, especially in the poultry and swine industries, the use of vaccines is considered crucial. In others, such as the cattle industries (cow-calf, feedlot, dairy), most vaccines are used with skepticism and with constant questions of poor cost/benefit ratios. The initial optimism of the new bioengineering companies as they looked at the animal vaccine market was based upon the extraordinarily large numbers of doses of vaccines produced. In 1985, in the United States, total production of all veterinary vaccine formulations was over 17 billion doses, of which about 93% consisted of poultry vaccines, representing about 39% of the total dollar value. The poultry vaccines were used to vaccinate 4.5 billion broilers, 45 million breeders, and 300 million layers. A total of 2.8 billion doses of Marek's disease vaccine were produced.

The initial enthusiasm of the new bioengineering companies waned in the realization that a very large number of doses of produced vaccines support a veterinary biologic industry with sales of only $300–400 million per year, of which about $118 million is in the poultry biologic industry. As an example of this fiscal reality, a common poultry vaccine is sold in a 25-ml vial containing 10,000 doses (delivered in broiler house water supply), which is sold for about $8.00. Of the nonpoultry vaccines, the largest market is that of rabies vaccines for pet animals. So, one question facing bioengineering companies is how to make a profit by expanding markets, not by just redividing them. To date, bioengineered vaccines for animals (and humans) that have reached the marketplace have been priced higher than conventional vaccines (e.g., swine pseudorabies vaccines). Larger markets will be built only if data and perceptions indicate to the farmer, rancher, and poultry producer that new bioengineered vaccines actually represent an improvement in value, that is, an improvement in cost/benefit equations.

The future role of vectored virus vaccines for animal diseases must be viewed in light of these realities. Products will only have value if they meet safety and efficacy demands, as set by government regulatory agencies and the public at large. If this can be achieved, then the great advantage of vectored virus vaccine products could lie in the low cost of manufacturing and ease of delivering them. Setbacks in the development of vectored virus vaccines for animal diseases have been caused by regulatory oversight of some animal experimentation and open field trials, but this will be corrected as regulatory details become clearer and more widely communicated.

In the end, overall benefit : cost : risk equations will determine the use

of particular vectored virus vaccines in preventing and controlling animal diseases. In our opinion, these equations will indicate an important role for many specific products that are now in development. The long-term future only promises more and more valuable products.

C. Vaccine Technology Transfer for Developing Countries

Some international agencies have stated that conventional vaccine production infrastructure can be leap-frogged and bioengineered vaccine production can be started from scratch in developing countries. Not everyone agrees. Several problems are: (1) there is too little opportunity (and funding) for training scientists from developing countries in the necessary technology, especially the technology used in manipulating genes and constructing recombinants by marker rescue; (2) most products and processes involving this technology are patented and limited in availability by proprietary ownership; and (3) foreign exchange shortages preclude most cooperative ventures between owners of proprietary products and processes and institutions in developing countries. One major problem is that no central international agency is charged with providing necessary leadership. An important activity of such an agency should be to develop standards. Currently, no standards exist for safety and efficacy. For example, with vectored vaccinia virus vaccines there is a need to monitor mutations in seed stocks as they undergo serial passage.

Several international agencies are funding the construction of vaccine factories in developing countries through various forms of bilateral and consortium agreements. In this context, recombinant DNA technology is proving to be particularly difficult to adapt to these arrangements because the technology demands extremely sophisticated facilities. Vectored poxvirus vaccines, in final form for production, may represent the first technology that is truly transferable to the Third World for local manufacture. Such technology transfer certainly must be tried.

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